京都大学	博士（工　　学）	氏名	王　文龍
論文題目	Studies on phenotypic and genotypic effects of xenoestrogens for integrated toxicity evaluation of environmental water（環境水の総合的毒性評価のための環境エストロゲンの表現型および遺伝子型影響に関する研究）		

（論文内容の要旨）

This dissertation is the first study to develop *in vitro* phenotype-based and *in vivo* genotype-based bioassay for integrated toxicity evaluation of xenoestrogens and indicates the usefulness for toxicity identification of xenoestrogens and environmental water samples including estrogenic effect, neurotoxicity, and DNA methylation.

Chapter 1 Introduction describes the background that motivates this research to be carried out, the objectives, the study scope and approach of this research.

Chapter 2 Brief introduction of xenoestrogen occurrence, their mode of mechanism, and their environmental risks. The current bioassay and promising bioassay for toxicity evaluation of xenoestrogens were also summarized for comparison.

Chapter 3 *In vitro* phenotypic analysis using MCF-7 cells was developed for integrated evaluation of estrogenic activities and other possible toxicity of whole water samples. The original study applying phenotypic analysis using MCF-7 cells for integrated toxicity evaluation of xenoestrogens, their mixture, and environmental water samples was exhibited. Morphological parameters of nuclei and cells (intensity, perimeters, major, minor, minor/major, area, form factors) were applied as multiple endpoints to catch more toxicity-pathways. A phenotypic database was constructed, by firstly using 26 organic chemicals with various toxicity pathways. The multivariate data analysis indicated that the morphological parameters addressed diverse biological pathways, which exhibits high-potential for integrated toxicity evaluation. Mixture effects on cellular morphologies were classified by toxicity-pathway, and the mixture with morphology-sensitive xenoestrogen induced similar morphological variation. These indicated the potential for toxicity identification using morphology-based analysis. Finally, the morphology-based analysis for integrated evaluation and identification of whole environmental samples in combination with conventional bioassays (cell viability, E-screen, and ELISA) and non-target chemical strategies (UPLC-qTOFMS) was applied. Phenotypic effects also showed significantly diverse results for whole water samples and exhibited high relation to organic chemicals structures based on non-target chemical analysis. A high correlation between nuclei intensity and estrogenic effect and morphological variation and overall chemicals structures of environmental water samples indicated the high potential for further chemical and toxicity identification. In all, *in vitro* morphology-based analysis showed correlation with toxicity-pathway and provided usuful information for integrated toxicity evaluation and identification of xenoestrogens in environmental water samples.

Chapter 4 *In vivo* genotypic analysis was performed using zebrafish for integrated evaluation of estrogenic effects and neurotoxicity of insecticides and environmental water samples. Multiple genetic

parameters including *ache*, *gabra1*, *gad2*, and *vtg1* were selected to provide an integrated evolution of the estrogenic effect and neurotoxicity. Firstly, genetic effects of each pesticide and their mixture were performed under no observed effect concentration (NOEC), which expressed a mechanism insight and the interactions of pesticides based on MoAs. Then, integrated toxicity of environmental water samples taken from Indonesia was evaluated based on multivariate data analysis approaches comparing with the mixture toxicity evaluation. The genotypic effect of insecticide exhibited diverse results. All the insecticide decreased the expression of *ache*, and chlorpyrifos induced the highest impact on *ache* expression due to the AchE inhibition mechanism. The *gabra1* and *gad2* expression significantly increased after exposure to permethrin. Imidacloprid and chlorpyrifos exhibited high estrogenic effects due to the significant activity on *vtg1* expression. Moreover, genotypic effect exhibited diverse results after exposed to water samples, agricultural wastewater induced neurotoxicity, and river water exhibited the significantly estrogenic effect. In conclusion, the multiple genetic parameters covered various toxicity pathways and provided an information-rich approach for integrated evaluation of neurotoxicity and estrogenic effects.

Chapter 5 *In vitro* morphology-based analysis of enhanced green fluorescent protein fused (EGFP) with methyl CpG-binding protein (MBD) was developed for evaluation of DNA methylation effect of xenoestrogens. The multiple morphological parameters of fluorescent EGFP were characterized and exhibited positive correlations between DNA methylation status and the phenotypic parameters comparing with the conventional DNA methylation analysis. Then, the DNA methylation effect of xenoestrogens including pesticides and estrogens was evaluated and classified with multivariate data analysis approaches. This study revealed novel insight for integrated toxicity evaluation of xenoestrogens. The (5-aza-dc) induced a decrease in intensity, area, and the count of EGFP-assembled granules, which demonstrated the positive correlations between DNA methylation status and the phenotypic parameters. With a high-throughput screening of environmental chemicals, it was found that imidacloprid, carbaryl, and o.p-DDT increased the parameters, indicating the induced DNA methylation. In conclusion, the granular sensitivity and area exhibited high correlations with DNA methylation, and the phenotypic analysis provided a high-throughput approach for DNA methylation evaluation.

Chapter 6 All the important findings are summarized and emphasized. Finally, future tasks and prospects are also described.

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（論文審査の結果の要旨）

本論文は、環境水の総合的毒性評価手法を開発するため、エストロゲン様物質の表現型および遺伝子型影響について実験およびフィールド調査によって検討を行ったものである。得られた主な成果は以下のとおりである。

1）環境水の毒性の総合的評価手法として、MCF-7 を用いた *in vitro* の表現型試験を開発するために、まず、様々な作用機序をもつ 26 種類の化学物質が、表現型へ与える影響を評価した。その結果、cell area や nuclei intensity といった表現型パラメータが、生物学的プロセス（細胞増殖や細胞間コミュニケーション、細胞死)や物理的因子（浸透圧等)と相関し、総合的毒性評価に使用できる可能性があることを示した。さらにこの表現型パラメータに基づく試験は、同じ作用機序をもつ化学物質を組み合わせて曝露した場合の毒性や、実際の環境サンプルの毒性を解析する場合にも有用であることを示した。また、実際の環境サンプルを用いた試験においては、nuclei intensity のパラメータは、E-screen や ELISA といった従来のバイオアッセイの結果と比較しても、サンプルのエストロゲン活性と相関がより高いことを示した。

2）ゼブラフィッシュの胎児を用いて、*in vivo* の遺伝子試験に基づく殺虫剤や環境水の毒性評価手法の開発を行った。その結果、*ache*、*gabra1*、*gad2*、*vtg1* といった遺伝子の発現を逆転写定量 PCR を用いて調べることで、サンプルの神経毒性やエストロゲン活性を評価することが可能であることを示した。

3）xenoestrogen による DNA メチル化に対する影響を評価するための、EGFP（enhanced green fluorescent protein fused)と MBD（methyl CpG-binding protein)を組み合わせた *in vitro* の表現型試験を開発した。その結果、granular sensitivity と granular area という 2 つのパラメータが、DNA メチル化と高い相関性を示し、本試験が DNA メチル化をハイスループットに評価する手法となり得ることを示した。

以上のように本論文は、本研究で提案した手法が環境水の総合的毒性評価手法として従来法に比べて有効であることを示すものであり、今後の河川や湖沼における水質管理計画の策定に大きく貢献するものであって、学術上、実際上寄与するところが少なくない。よって、本論文は博士（工学）の学位論文として価値あるものと認める。また、平成３０年１月２４日、論文内容とそれに関連した事項について試問を行って、申請者が博士後期課程学位取得基準を満たしていることを確認し、合格と認めた。